Biochimica et Biophysica Acta 1798 (2010) 1164–1171

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Sodium-coupled electrogenic transport of pyroglutamate (5-oxoproline) via SLC5A8, a monocarboxylate transporter

Seiji Miyauchi, Elangovan Gopal, Ellappan Babu, Sonne R. Srinivas, Yoshiyuki Kubo, Nagavedi S. Umapathy, Santoshanand V. Thakkar, Vadivel Ganapathy, Puttur D. Prasad *

*Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA 30912, USA*

ARTICLE INFO

*Article history:*
Received 6 November 2009
Received in revised form 26 February 2010
Accepted 1 March 2010
Available online 6 March 2010

*Keywords:*
Pyroglutamate
Renal reabsorption
SLC5A8
Monocarboxylate transporter

ABSTRACT

Pyroglutamate, also known as 5-oxoproline, is a structural analog of proline. This amino acid derivative is a byproduct of glutathione metabolism, and is reabsorbed efficiently in kidney by $Na^+$-coupled transport mechanisms. Previous studies have focused on potential participation of amino acid transport systems in renal reabsorption of this compound. Here we show that it is not the amino acid transport systems but instead the $Na^+$-coupled monocarboxylate transporter SLC5A8 that plays a predominant role in this reabsorptive process. Expression of cloned human and mouse SLC5A8 in mammalian cells induces $Na^+$-dependent transport of pyroglutamate that is inhibitable by various SLC5A8 substrates. SLC5A8-mediated transport of pyroglutamate is saturable with a Michaelis constant of $0.36 \pm 0.04$ mM. $Na^+$-activation of the transport process exhibits sigmoidal kinetics with a Hill coefficient of $1.8 \pm 0.4$, indicating involvement of more than one $Na^+$ in the activation process. Expression of SLC5A8 in *Xenopus laevis* oocytes induces $Na^+$-dependent inward currents in the presence of pyroglutamate under voltage-clamp conditions. The concentration of pyroglutamate necessary for induction of half-maximal current is $0.19 \pm 0.01$ mM. The $Na^+$-activation kinetics is sigmoidal with a Hill coefficient of $2.3 \pm 0.2$. Ibuprofen, a blocker of SLC5A8, suppressed pyroglutamate-induced currents in SLC5A8-expressing oocytes; the concentration of the blocker necessary for causing half-maximal inhibition is $14 \pm 1$ μM. The involvement of SLC5A8 can be demonstrated in rabbit renal brush border membrane vesicles by showing that the $Na^+$-dependent uptake of pyroglutamate in these vesicles is inhibitable by known substrates of SLC5A8. The $Na^+$ gradient-driven pyroglutamate uptake was stimulated by an inside-negative $K^+$ diffusion potential induced by valinomycin, showing that the uptake process is electrogenic.



## 1. Introduction

Pyroglutamate (also known as 5-oxoproline or pyrrolidone carboxylate) is a cyclic lactam of glutamate. It is an intermediate in γ-glutamyl cycle associated with glutathione metabolism where it is generated by the action of γ-glutamyl cyclotransferase on γ-glutamyl amino acids and γ-glutamyl peptides [1]. It is also produced by enzymatic hydrolysis of N-terminal pyroglutamate present in a variety of peptides and proteins [2]. Recent studies have shown that pyroglutamate may have important biologic functions in the regulation of amino acid transport [3,4]. Pyroglutamate is present in free form in all animal tissues, plasma, and urine. The plasma levels vary in the range of 10–40 μM under normal physiologic conditions [5]. It is absorbed very effectively in the kidney. Normal urinary excretion is ~250 μmol/day in humans [6], which shows that the fractional reabsorption of pyroglutamate in the kidney exceeds 85%. Excretion of pyroglutamate in urine increases several-fold in patients with genetic defects in glutathione metabolism associated with the deficiency of either glutathione synthetase or 5-oxoprolinase [7,8]. The increased excretion in these diseases can be explained by the overproduction of 5-oxoproline, which exceeds the absorption maximum in the kidney.

The effective reabsorption of pyroglutamate in the kidney indicates the presence of efficient transport systems for this metabolite in the apical membrane of renal tubular cells. Several early studies with purified renal brush border membrane vesicles have provided evidence for a $Na^+$-coupled electrogenic transport process for pyroglutamate in the kidney, and for the involvement of amino acid transport systems in the process [9–12]. Neutral amino acids share the transport process with pyroglutamate. However, none of the amino acids examined was able to block pyroglutamate uptake in these membrane vesicles completely. In fact, at physiological concentrations, none of the neutral amino acids tested was able to inhibit pyroglutamate uptake by more than 10%. These findings suggest that there are as yet unidentified transport systems contributing to renal reabsorption of this compound.

* Corresponding author. Tel.: +1 706 721 1761; fax: +1 706 721 6608.
*E-mail address:* pprasad@mcg.edu (P.D. Prasad).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.03.002

The present studies were undertaken to evaluate the role of SLC5A8 in the transport of pyroglutamate. SLC5A8 is a $Na^+$-coupled transporter for a variety of monocarboxylates such as lactate and pyruvate [13], short-chain fatty acids [14–16], ketone bodies [17], nicotinate and its structural analogs [18,19], and benzoate and its derivatives [20]. It is expressed in the apical membrane of renal tubular epithelial cells throughout the proximal tubule [21]. It plays an obligatory role in the renal reabsorption of lactate [22,23]. What prompted the present study was the ability of SLC5A8 to transport nicotinate and the structural similarity between nicotinate and pyroglutamate. Even though pyroglutamate is an amino acid derivative, it exists predominantly as an anionic monocarboxylate at physiological pH. This indicated that pyroglutamate may be recognized by SLC5A8 as a transportable substrate. The present studies show that it is indeed the case.

## 2. Materials and methods

### 2.1. Materials

[$^{14}$C]Nicotinate was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [$^3$H]Pyroglutamate was purchased from Moravek (Brea, CA, USA). Unlabeled monocarboxylates and valinomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA) Human SLC5A8 and mouse Slc5a8 were originally cloned from human intestine and mouse kidney, respectively [13,14].

### 2.2. Transport of pyroglutamate via SLC5A8 in a mammalian cell heterologous expression system

Human, mouse, and rat SLC5A8 cDNAs were expressed in the human retinal pigment epithelial cell line HRPE using the vaccinia virus expression technique [14]. The transport function of SLC5A8 was monitored by the uptake of [$^{14}$C]nicotinate (15 μM) in the presence of NaCl, and the interaction of pyroglutamate with the transporter was investigated by studying the effect of pyroglutamate on SLC5A8-mediated nicotinate uptake. HRPE cells transfected with empty vector were used to measure background transport activity. Uptake measurements in vector- and cDNA-transfected cells were made in parallel under identical experimental conditions. The SLC5A8-specific transport was determined by subtracting the transport values measured in vector-transfected cells from the transport values measured in cDNA-transfected cells. The uptake buffer was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. The influence of $Na^+$ on the transport process was assessed by monitoring the uptake of pyroglutamate in a $Na^+$-free buffer, which was prepared by substituting NaCl in the uptake buffer with *N*-methyl-D-gluconate (NMDG) chloride isoosmotically. The transport of pyroglutamate via SLC5A8 was also studied directly by using [$^3$H]pyroglutamate (0.25 μM). Saturation kinetics of pyroglutamate uptake was studied by analyzing the data according to the Michaelis–Menten equation to determine the kinetic parameters Michaelis constant ($K_t$) and maximal velocity ($V_{max}$). Substrate selectivity was investigated by determining the ability of various monocarboxylates to compete with pyroglutamate for the uptake process. $Na^+$-activation kinetics was investigated by monitoring SLC5A8-specific pyroglutamate uptake in the presence of increasing concentrations of $Na^+$. The kinetic parameters, the Hill coefficient ($h$, number of $Na^+$ ions involved in the activation process) and $K_{0.5}$ ($Na^+$ concentration necessary for half-maximal activation of pyroglutamate uptake) were calculated by fitting the Hill equation to the experimental data. Results are given as means ± S. E. ($n$ = 4–6).

### 2.3. SLC5A8-mediated transport of pyroglutamate using the Xenopus laevis oocyte expression system

Capped cRNA from human SLC5A8 cDNA (cloned in pGH19, an *X. laevis* oocyte expression vector) was synthesized using the mMESSAGE-mMACHINE kit (Ambion, Austin, TX, USA). Mature oocytes from *X. laevis* were isolated by treatment with collagenase A (1.6 mg/ml), manually defolliculated and maintained at 18 °C in modified Barth's medium, supplemented with 25 μg/ml gentamicin as described previously [14]. Oocytes were injected with 50 ng of cRNA. Water-injected oocytes served as controls. The transport function of the heterologously expressed SLC5A8 was monitored by two different techniques, first by measuring the uptake of [$^3$H]pyroglutamate and second by using an electrophysiological method. Uptake of [$^3$H]pyroglutamate (0.8 μM) in water-injected and cRNA-injected oocytes was measured for 60 min in the presence or absence of $Na^+$ (i.e., NaCl buffer or NMDG chloride buffer) as described previously [14]. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method. Oocytes were perifused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 7.5), followed by the same buffer containing pyroglutamate or other structurally compounds. Once the inward currents plateaued off, the uptake was terminated by perifusing the oocytes with a buffer in which NaCl was isoosmotically replaced with NMDG chloride. The membrane potential was clamped at −50 mV. The differences between the steady-state currents measured in the presence and absence of substrates were considered as the substrate-induced currents. In the analysis of the saturation kinetics of substrate-induced currents, the kinetic parameter $K_{0.5}$ (i.e., substrate concentration necessary for induction of half-maximal current) was calculated by fitting the Michaelis–Menten equation to the values of the substrate-induced currents. The $Na^+$-activation kinetics of substrate-induced currents was analyzed by measuring the substrate-specific currents in the presence of increasing concentrations of $Na^+$. The data for the $Na^+$-dependent currents were analyzed according to the Hill equation to determine the Hill coefficient ($h$, the number of $Na^+$ ions involved in the activation process) and $K_{0.5}$ for $Na^+$ (i.e., concentration of $Na^+$ necessary for half-maximal activation). Since expression levels varied significantly from oocyte to oocyte, kinetic analyses were done by normalizing the expression levels. This was done by taking the maximally induced SLC5A8-specific current in each kinetic experiment in individual oocytes as 1. To investigate the current–voltage ($I$–$V$) relationship, step changes in membrane potential were applied, each for a duration of 100 ms in 20-mV increments. Each experiment was repeated with four different oocytes. The influence of ibuprofen, a blocker of SLC5A8 transport function [19], was studied by analyzing the effect of this compound on pyroglutamate-induced currents in SLC5A8-expressing oocytes. Each experiment was repeated with at least 3 or 4 oocytes, and the data are given as means ± S. E.

### 2.4. Transport of pyroglutamate in rabbit kidney brush border membrane vesicles

Brush border membrane vesicles were prepared from rabbit kidney cortex by the $Mg^{2+}$-EGTA precipitation technique as described previously [9–11]. The vesicles were preloaded with 25 mM Hepes/Tris buffer (pH 7.5), containing 150 mM potassium gluconate. Uptake of [$^3$H]pyroglutamate (1 μM) in these vesicles was measured with a 15 s incubation in the presence of a NaCl-containing buffer (25 mM Hepes/Tris, pH 7.5, with 150 mM NaCl). The dependence of pyroglutamate uptake on $Na^+$ was assessed by monitoring uptake in a $Na^+$-free buffer in which NaCl was substituted with KCl isoosmotically. The ability of lactate, pyruvate, nicotinate, propionate and butyrate (substrates for SLC5A8), and ibuprofen (blocker of SLC5A8) to inhibit pyroglutamate uptake process was assessed in

these membrane vesicles. Conversely, the activity of Slc5a8 in these vesicles was monitored by measuring $Na^+$-dependent uptake of [$^{14}$C] nicotinate (25 μM). This was done by measuring uptake in the presence and absence of $Na^+$ and then calculating the difference between the two values. The interaction of pyroglutamate and other structurally related compounds with Slc5a8 was assessed by the ability of these compounds to inhibit $Na^+$-dependent nicotinate uptake. The experiments were performed twice with two different preparations of membrane vesicles, and each experiment was done in triplicate. Data are given as means ± S. E.

### 2.5. Statistical analysis

Student's *t* test was used to determine the significance of differences between the means from two groups. Statistical significance among means of more than two groups was analyzed by one-way analysis of variance (ANOVA). A $p<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Structural relationship between nicotinate and pyroglutamate

Pyroglutamate, also known as 5-oxoproline, represents the cyclized form of glutamate with the amino group and the carboxylate group forming an intramolecular amide linkage. In proteins and peptides, pyroglutamate is formed when N-terminal glutamine cyclizes with concomitant deamidation. The presence of the intramolecular amide bond in pyroglutamate prevents protonation of the imino group, and consequently pyroglutamate has only the carboxylic acid group as the ionizable moiety ($pK_a = 4.2$). Therefore, it exists predominantly as a monovalent anion at physiologic pH. In contrast, proline, which does not have the intramolecular amide bond, exists predominantly as a zwitterion at physiologic pH due to protonation of the imino group ($pK_a = 9.9$) and deprotonation of the carboxylic acid group ($pK_a = 3.9$). Pyroglutamate bears significant structural resemblance to nicotinate, which also exists as a monovalent anion at physiologic pH. The chemical structures of glutamate, glutamine, proline, pyroglutamate, and nicotinate are given in Fig. 1.

### 3.2. Transport of pyroglutamate by SLC5A8 in a heterologous expression system with a mammalian cell line

We first used a mammalian cell expression system to investigate the transport of pyroglutamate via SLC5A8. We expressed the cloned human SLC5A8 in the human retinal pigment epithelial cell line HRPE heterologously using the vaccinia virus expression technique. Cells transfected with vector alone served as the control. The activity of SLC5A8 was monitored by [$^{14}$C]nicotinate uptake in the presence of $Na^+$. The uptake of nicotinate in SLC5A8 cDNA-transfected cells was 6-fold higher than in vector-transfected cells (data not shown). SLC5A8-specific uptake was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. The uptake was linear up to 7.5 min. We studied the effects of pyroglutamate, glutamate, glutamine, and proline on SLC5A8-specific nicotinate uptake (Fig. 2). When present at 5 mM, pyroglutamate caused 80–90% inhibition of SLC5A8-specific [$^{14}$C]nicotinate uptake. This inhibition was similar to the inhibition caused by 5 mM unlabeled nicotinate. Glutamate and glutamine did not inhibit the uptake. Proline stimulated the uptake to a small, but significant extent ($p<0.01$). These data show that pyroglutamate interacts with SLC5A8 and competes with nicotinate for the uptake process.

We then assessed the transport of pyroglutamate via SLC5A8 directly by monitoring the uptake of [$^3$H]pyroglutamate in the same heterologous expression system. Uptake of pyroglutamate in SLC5A8-expressing cells was several-fold greater than in vector-transfected cells (Fig. 3A). This uptake was $Na^+$-dependent; there was no detectable difference between uptake in SLC5A8-expressing cells and vector-transfected cells when monitored in the absence of $Na^+$. Similar results were obtained with mouse Slc5a8. The transport of pyroglutamate via SLC5A8 was inhibitable by the known substrates of the transporter (Fig. 3B). This includes short-chain fatty acids (acetate, propionate, and butyrate), nicotinate, pyruvate, and lactate. Unlabeled pyroglutamate also competed with [$^3$H]pyroglutamate for the uptake process. Glutamine had no effect on SLC5A8-mediated pyroglutamate uptake ($p>0.05$) whereas proline and glutamate caused significant inhibition ($p<0.01$). However, the inhibition caused by proline and glutamate was much smaller than the inhibition caused by SLC5A8 substrates.

The transport of pyroglutamate via SLC5A8 was saturable, with a Michaelis constant of 0.36 ± 0.04 mM (Fig. 4A). $Na^+$-activation kinetics indicated that the relationship between SLC5A8-mediated pyroglutamate uptake and $Na^+$ concentration was sigmoidal (Fig. 4B), suggesting involvement of more than one $Na^+$ in the activation process. The data were fit to the Hill equation and the Hill coefficient, which is the number of $Na^+$ ions interacting with the carrier was calculated. The value was 1.8 ± 0.4, which indicates that for every molecule of pyroglutamate transported, 2 $Na^+$ ions are also cotransported.

### 3.3. Transport of pyroglutamate by SLC5A8 in a heterologous expression system with X. laevis oocytes

We also investigated the transport of pyroglutamate via human SLC5A8 using a different functional expression system. We expressed the cloned human SLC5A8 in *X. laevis* oocytes by injection of cRNA. Water-injected oocytes served as controls. First, we monitored the uptake of [$^3$H]pyroglutamate in water-injected and SLC5A8 cRNA-injected oocytes. The uptake was ~60-fold higher in cRNA-injected oocytes than in water-injected oocytes (Fig. 5A). This uptake was completely $Na^+$-dependent; there was no significant difference in uptake between water-injected oocytes and SLC5A8-expressing oocytes when measured in the absence of $Na^+$ (6.7 ± 0.7 pmol/

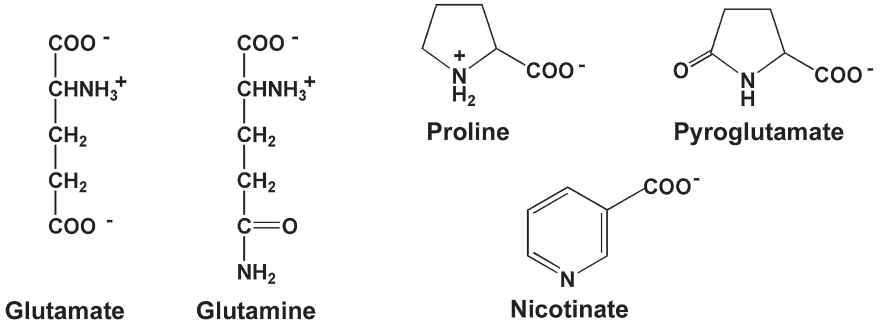


**Fig. 1.** Structures of glutamate, glutamine, proline, pyroglutamate, and nicotinate.

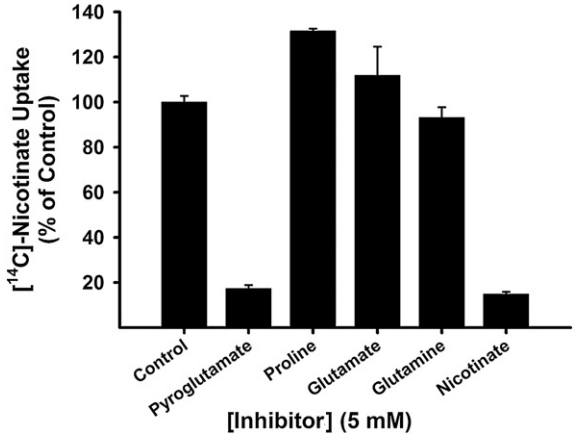


**Fig. 2.** Inhibition of SLC5A8-mediated nicotinate uptake by pyroglutamate. HRPE cells were transfected with either pSPORT alone or human SLC5A8 cDNA. The expression of the transfected cDNA was facilitated by the vaccinia virus expression technique. Uptake of [$^{14}$C]nicotinate (15 μM) was monitored with a 15 min incubation, and SLC5A8-specific transport of nicotinate was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. Uptake measurements were made in the absence or presence of pyroglutamate, proline, glutamate, glutamine, and nicotinate (5 mM). Uptake measured in the absence of inhibitors was taken as 100% (control), and uptake in the presence of inhibitors is given as percent of this control value. The experiment was repeated two times and each experiment was done in triplicate. Data represent means ± S. E.

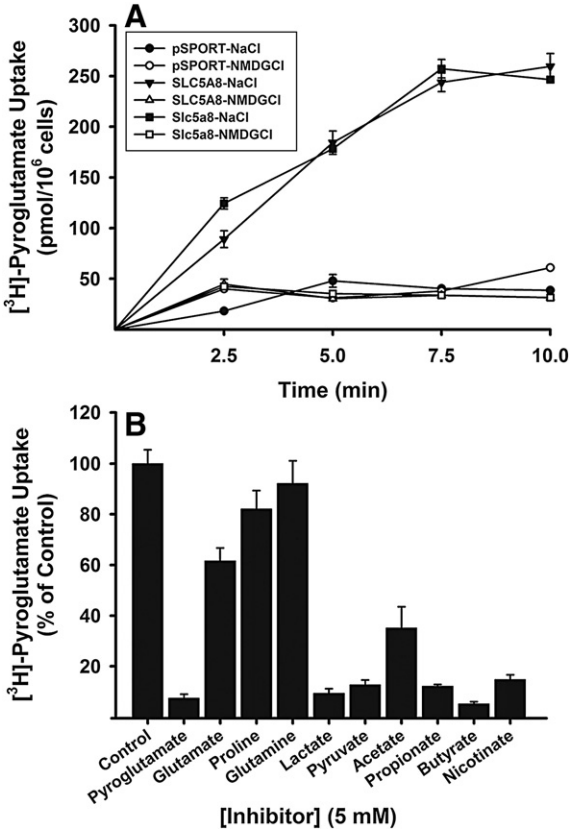


**Fig. 3.** Characteristics of SLC5A8-mediated pyroglutamate uptake. (A) Human SLC5A8 and mouse Slc5a8 were expressed functionally in HRPE cells using the vaccinia virus expression technique. Cells transfected with vector alone served as controls. Uptake of [$^3$H]pyroglutamate (0.25 μM) was measured for varying time periods in the presence (NaCl) or absence of $Na^+$ (NMDG chloride). The experiment was carried out in triplicate and repeated twice. The data represent means ± S. E. (B) Inhibition of SLC5A8-mediated pyroglutamate uptake by amino acids and monocarboxylates. HRPE cells were transfected with human SLC5A8 cDNA or pSPORT. Vaccinia virus expression technique was used to facilitate the expression of the transfected cDNA. Uptake of [$^3$H] pyroglutamate (0.25 μM) was measured with 15 min incubation in parallel in vector-transfected cells and in cDNA-transfected cells, and SLC5A8-specific transport was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. Uptake measurements were made in the absence or presence of various amino acids and monocarboxylates (5 mM). Uptake measured in the absence of inhibitors was taken as 100% (control), and uptake in the presence of inhibitors is given as percent of this control value. The experiment was done in triplicate and repeated twice. Data are given as means ± S. E.

oocyte/h in the presence of $Na^+$; 7.1 ± 1.7 pmol/oocyte/h in the absence of $Na^+$; $p > 0.05$).

We have used previously an electrophysiological approach to characterize the transport function of SLC5A8. Since the transporter transports monocarboxylates with a negative charge along with more than one $Na^+$ per transport cycle, the transport process is electrogenic resulting in depolarization of the membrane potential. Present studies have shown that SLC5A8-mediated pyroglutamate transport is $Na^+$-dependent with involvement of more than one $Na^+$. Pyroglutamate exists mostly as a monovalent anion under physiologic conditions; therefore, transport of this monovalent anion with more than one $Na^+$ per transport cycle is expected to result in the transfer of one net positive charge into oocytes, thus causing membrane depolarization. Therefore, we used the two-microelectrode voltage-clamp technique to investigate the electrogenic nature of SLC5A8-mediated pyroglutamate transport. Superfusion of SLC5A8-expressing oocytes with pyroglutamate (5 mM) induced marked inward currents under voltage-clamp conditions (Fig. 5B). Such currents were not detectable in water-injected oocytes (data not shown). The pyroglutamate-induced currents in SLC5A8-expressing oocytes were absolutely dependent on $Na^+$ because the currents were not detectable under $Na^+$-free conditions. The anion chloride had no effect on pyroglutamate transport via SLC5A8. The magnitude of the inward current induced by pyroglutamate (5 mM) in the presence of $Na^+$ but in the absence of $Cl^-$ was 219 ± 10 nA (3 different oocytes). Glutamate, glutamine, and proline did not induce currents via SLC5A8, indicating that while pyroglutamate is a transportable substrate for SLC5A8, glutamate, glutamine, and proline are not. Current–voltage (*I–V*) relationship for nicotinate- and pyroglutamate-induced currents indicates that the rate of transport is dependent on membrane potential as expected of an electrogenic process. The higher the membrane potential, the greater were the currents induced by nicotinate and pyroglutamate in SLC5A8-expressing oocytes (Fig. 5C). The maximal currents induced by nicotinate (5 mM) and pyroglutamate (5 mM) at −50 mV are 323 ± 13 nA and 226 ± 7 nA (4 different oocytes).

We then investigated the kinetic features of pyroglutamate transport via SLC5A8 using the same electrophysiological approach. Here we used pyroglutamate-induced currents as the measure of transport rate. The transport process was saturable, with a Michaelis constant of 0.19 ± 0.01 mM (Fig. 6A). $Na^+$-activation kinetics indicated a sigmoidal relationship between pyroglutamate-induced currents and $Na^+$ concentration (Fig. 6B). Pyroglutamate-induced currents reached a maximal steady state at 100 mM $Na^+$. Determination of the Hill coefficient using the Hill equation (Fig. 6B, inset) gave a value of 2.3 ± 0.2 for the Hill coefficient, corroborating the data obtained with the mammalian expression system that at least 2 $Na^+$ are involved in the activation process. The $Na^+$:pyroglutamate stoichiometry of 2:1 provides the basis of the electrogenic nature of the transport process.

Our previous studies have shown that ibuprofen is a blocker of SLC5A8-mediated transport process [19]. Here we studied the effects of this SLC5A8-specific blocker on SLC5A8-mediated pyroglutamate transport. We monitored pyroglutamate transport electrophysiologically with superfusion of SLC5A8-expressing oocytes with pyroglutamate (1 mM) in the presence of increasing concentrations of ibuprofen (Fig. 7A). Pyroglutamate-induced inward currents in the presence of $Na^+$ under voltage-clamp conditions. Ibuprofen decreased the magnitude of these currents in a dose-dependent manner. Kinetic analysis of this effect showed that the concentration of

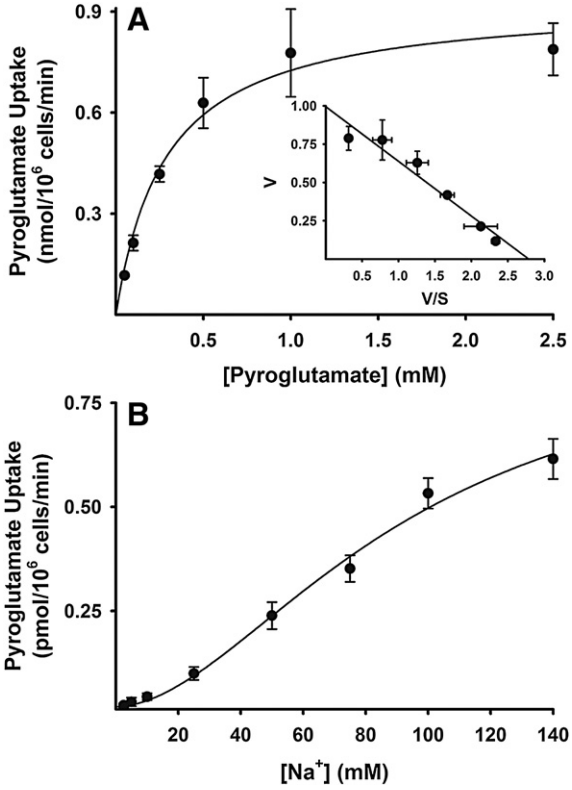


**Fig. 4.** Saturation kinetics (A) and $Na^+$-activation kinetics (B) of SLC5A8-mediated pyroglutamate transport. (A) The vaccinia virus technique was used to express human SLC5A8 in HRPE cells. Vector-transfected cells served as control. Uptake of pyroglutamate was measured with a 7.5 min incubation in the presence of increasing concentrations of pyroglutamate (50–2500 μM). The amount of [$^3$H]pyroglutamate was kept constant at 0.25 μM. SLC5A8-specific uptake was calculated by subtracting the uptake in vector-transfected cells from the uptake in cDNA-transfected cells. Only the SLC5A8-specific uptake was used for kinetic analysis. Inset: Eadie–Hofstee plot. (B) SLC5A8-specific pyroglutamate uptake was measured in HRPE cells as described above. Measurements were made in the presence of increasing concentrations of $Na^+$ (2.5–140 mM) with a fixed concentration of $Cl^-$ (140 mM). $Na^+$ concentrations were varied by appropriately substituting NaCl with NMDG chloride. Data represent means ± S. E. for six determinations.

ibuprofen needed for half-maximal blockade of the currents was 14 ± 1 μM (Fig. 7B).

### 3.4. Studies with rabbit renal brush border membrane vesicles

Slc5a8 is expressed exclusively in the brush border membrane of proximal tubular cells in mouse kidney where it mediates active $Na^+$-coupled reabsorption of lactate and nicotinate [21]. Therefore, the transport function of Slc5a8 can be studied using purified renal brush border membrane vesicles. This provides an opportunity to investigate the transport of pyroglutamate via Slc5a8 in a native state. We first studied the uptake of nicotinate in rabbit renal brush border membrane vesicles as a measure of Slc5a8 activity. With 15 s incubation, the uptake of nicotinate (15 μM) in these vesicles was 7-fold greater in the presence of NaCl-containing medium than in the presence of KCl-containing medium, indicating $Na^+$-coupled transport of nicotinate. We then monitored the effects of pyroglutamate (5 mM) on nicotinate (15 μM) uptake in the presence and absence of $Na^+$. Pyroglutamate inhibited specifically the $Na^+$-dependent uptake (75%; $p<0.05$) (Fig. 8A). There was no significant effect on $Na^+$-independent uptake (data not shown). At a concentration of 5 mM, the amino acids proline, glutamate, and glutamine also caused significant inhibition of nicotinate uptake (30–40%; $p<0.05$). We also studied the uptake of pyroglutamate directly in these vesicles. The time course of uptake in the presence of $Na^+$ showed the typical overshoot phenomenon; the uptake was maximal at 30 s and then reached equilibrium at 60 min (data not shown). The uptake at the peak of the overshoot was five times greater than the equilibrium value, indicating transient concentrative uptake. The uptake in the absence of $Na^+$ (i. e., KCl-containing medium) was slower than in the presence of $Na^+$ with no evidence of overshoot. The equilibrium values were however similar both in the presence and absence of $Na^+$. These are typical characteristics of any $Na^+$-coupled active uptake process in purified renal brush border membrane vesicles. We then assessed the effects of known transportable substrates for Slc5a8 and ibuprofen, a blocker of SLC5A8, on pyroglutamate uptake (Fig. 8B). The uptake of pyroglutamate (1 μM) was inhibited by ~90% in the presence of these compounds (5 mM). These data demonstrate that pyroglutamate and these compounds compete for a common $Na^+$-coupled transport process in the renal brush border membrane. The effect of a valinomycin-induced inside-negative membrane potential on $Na^+$-dependent pyroglutamate uptake was also investigated. Uptake of pyroglutamate was stimulated by 12 ± 3% ($p<0.05$) in the presence of valinomycin (10 μM final concentration), compared with pyroglutamate uptake in the absence of valinomycin (data not shown). These data show that $Na^+$ gradient-driven pyroglutamate uptake is electrogenic and that the uptake is associated with a net transfer of positive charge across the membrane.

## 4. Discussion

Here we demonstrate for the first time the involvement of the $Na^+$-coupled monocarboxylate transporter SLC5A8 (also known as SMCT1) in the renal reabsorption of the amino acid derivative pyroglutamate. Because of the structural resemblance of pyroglutamate to proline, previous studies focused on participation of amino acid transport systems in the renal transport of this compound [9,10]. These studies did show that neutral amino acids interact with the transport system that is responsible for pyroglutamate uptake in renal brush border membrane vesicles. But, the maximum inhibition of pyroglutamate uptake caused by any of the amino acids is ~60%, indicating potential involvement of other transport systems in the uptake process.

Lactate is a monocarboxylate with no structural resemblance to pyroglutamate. This metabolite is reabsorbed in the kidney by an effective $Na^+$-coupled transport system [24–27]. Because of the lack of significant structural similarity between lactate and pyroglutamate, interaction between these two compounds during the renal reabsorptive process was never suspected. The transporter responsible for lactate reabsorption in the kidney has now been cloned [13]. This transporter, known as SMCT1 (i.e., Sodium-coupled MonoCarboxylate Transporter 1), mediates $Na^+$-coupled transport of lactate with high affinity. It is expressed in the apical membrane of the proximal tubular cells [21]. Absence of the transporter in the kidney leads to increased urinary excretion of lactate [22,23], providing evidence for an essential role of this transporter for absorption of lactate from glomerular filtrate. Studies with purified brush border membrane vesicles have indicated that the renal transport system for lactate also transports other monocarboxylates such as nicotinate and pyrazinoate [28]. These findings led us to test the ability of the cloned SLC5A8 to transport nicotinate [18,20]. We found that SLC5A8, which is a $Na^+$-coupled transporter for linear-chain monocarboxylates such as lactate, pyruvate, butyrate, propionate and acetate, also functions as a $Na^+$-coupled transporter for nicotinate, pyrazinoate, benzoate, salicylate, and 5-aminosalicylate [18,20]. This was interesting because it became evident for the first time that SLC5A8 can accept as substrates not only linear-chain monocarboxylates but also monocarboxylates with aromatic and heterocyclic ring structures. This new insight into the structural features of SLC5A8 substrates led us to revisit the transport mechanisms responsible for renal reabsorption of pyroglutamate because of the structural similarity between pyroglutamate and nicotinate.

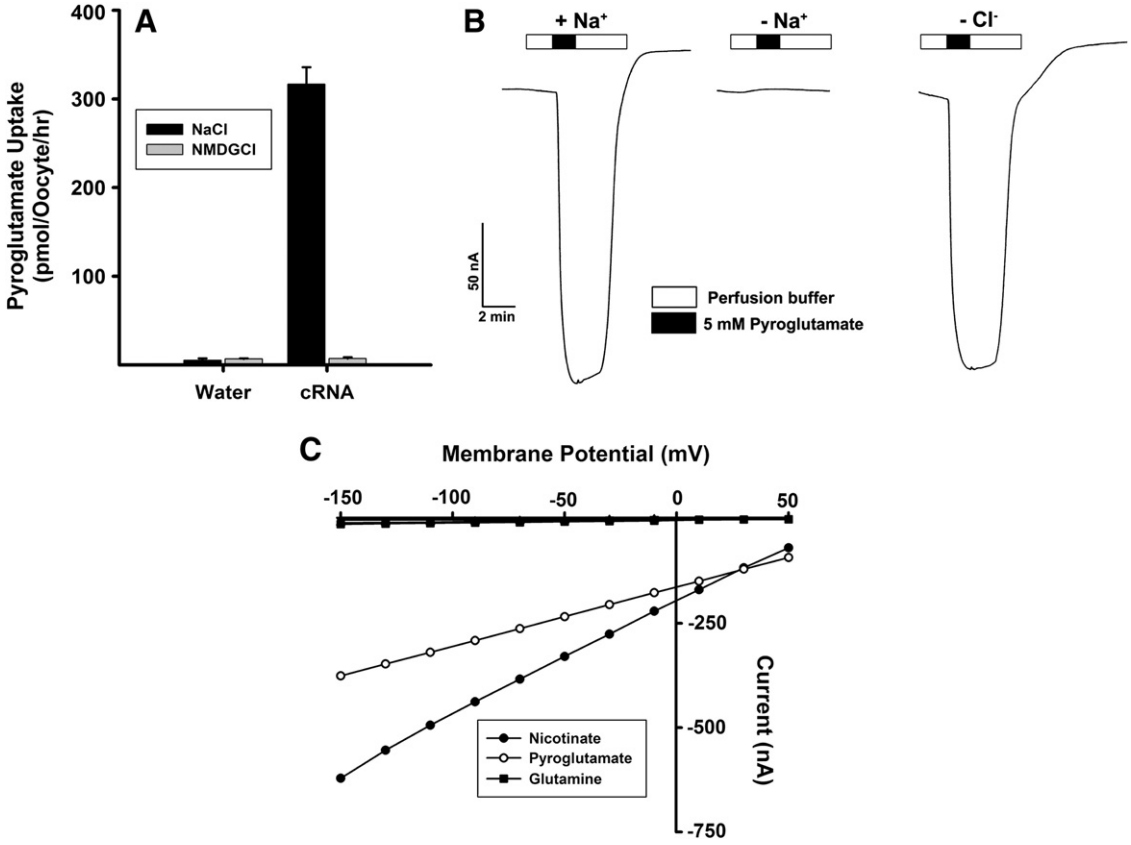


**Fig. 5.** Demonstration of SLC5A8-mediated pyroglutamate transport in *X. laevis* oocyte expression system. (A) Uptake of [$^3$H]pyroglutamate (0.8 μM) was measured in water-injected oocytes and in SLC5A8 cRNA-injected oocytes in the presence or absence of $Na^+$. Data represent means ± S. E. for uptake in 10 different oocytes. (B) SLC5A8 cRNA-injected oocytes were superfused with 5 mM pyroglutamate in the presence of NaCl ($+Na^+$), NMDG chloride ($-Na^+$), or sodium gluconate ($-Cl^-$). Currents were monitored by the two-microelectrode voltage-clamp technique. (C) Currents induced by pyroglutamate, nicotinate, proline, glutamate, and glutamine (5 mM) were monitored in SLC5A8 cRNA-injected oocytes using the two-microelectrode voltage-clamp technique with varying membrane potential. Since the individual points for proline, glutamate and glutamine overlap and are not distinguishable, only the data points for glutamine are shown.

Here we showed that SLC5A8 does indeed transport pyroglutamate very effectively. We demonstrated this phenomenon with human SLC5A8 as well as with mouse Slc5a8. Expression of cloned human and mouse SLC5A8 in mammalian cells induced $Na^+$-dependent transport of pyroglutamate that was inhibitable by various SLC5A8 substrates, including lactate and nicotinate. SLC5A8-mediated transport of pyroglutamate was saturable with a Michaelis constant of 0.36 ± 0.04 mM. $Na^+$-activation of the transport process exhibited sigmoidal kinetics with a Hill coefficient of 1.8 ± 0.4, indicating the involvement of more than one $Na^+$ in the activation process. The ability of SLC5A8 to transport pyroglutamate was also studied using a different expression system. Expression of SLC5A8 in *X. laevis* oocytes induced $Na^+$-dependent inward currents in the presence of pyroglutamate under voltage-clamp conditions. The concentration of pyroglutamate necessary for induction of half-maximal current was 0.19 ± 0.01 mM. The $Na^+$-activation kinetics was sigmoidal with a Hill coefficient of 2.3 ± 0.2. Ibuprofen, a specific blocker of SLC5A8, suppressed pyroglutamate-induced currents in SLC5A8-expressing oocytes; the concentration of the blocker necessary for causing half-maximal inhibition was 14 ± 1 μM. We also studied the involvement of the $Na^+$-coupled lactate transport system in the kidney in the transport of pyroglutamate directly using purified rat renal brush border membrane vesicles. The $Na^+$-dependent uptake of pyroglutamate in these vesicles was inhibitable by lactate.

It was interesting to note that the amino acids proline, glutamate, and glutamine caused 30–40% inhibition of nicotinate uptake in renal brush border membrane vesicles. To the best of our knowledge, $Na^+$-coupled transport of nicotinate occurs only via SLC5A8. In *X. laevis* oocytes expressing SLC5A8, there was no evidence of transport of these amino acids via the transporter because none of these amino acids induced significant inward currents in these oocytes. Therefore, SLC5A8 does not recognize amino acids as substrates. The findings that these amino acids inhibited $Na^+$-coupled nicotinate uptake in renal brush border membrane vesicles suggest that the observed inhibitory effects may be indirect due to amino acid-induced dissipation of transmembrane electrochemical $Na^+$ gradient. The renal brush border membrane possesses several $Na^+$-coupled electrogenic transport systems for proline, glutamate, and glutamine. This includes SLC6A19 ($B^0$AT1), SLC6A20 (SIT1), and SLC1A1 (EAAT3). The presence of these amino acids at high concentrations (e.g., 5 mM) in the medium is expected to reduce the transmembrane concentration gradient for $Na^+$ as well as generate an inside-positive membrane potential in renal brush border membranevesicles due to transport via these $Na^+$-coupled electrogenic amino acid transporters. This will reduce the driving force for the transport of pyroglutamate via SLC5A8. We speculate that the observed 30–40% inhibition of SLC5A8 transport function by proline, glutamate, and glutamine might be at least partly due to this indirect effect. In contrast to these amino acids, other substrates of SLC5A8 inhibit 80–90% of $Na^+$-coupled pyroglutamate transport in these membrane vesicles. Based on these findings, we conclude that SLC5A8 is responsible for a predominant portion of renal reabsorption of pyroglutamate and that the amino acid transport systems play relatively only a minor role in the process.

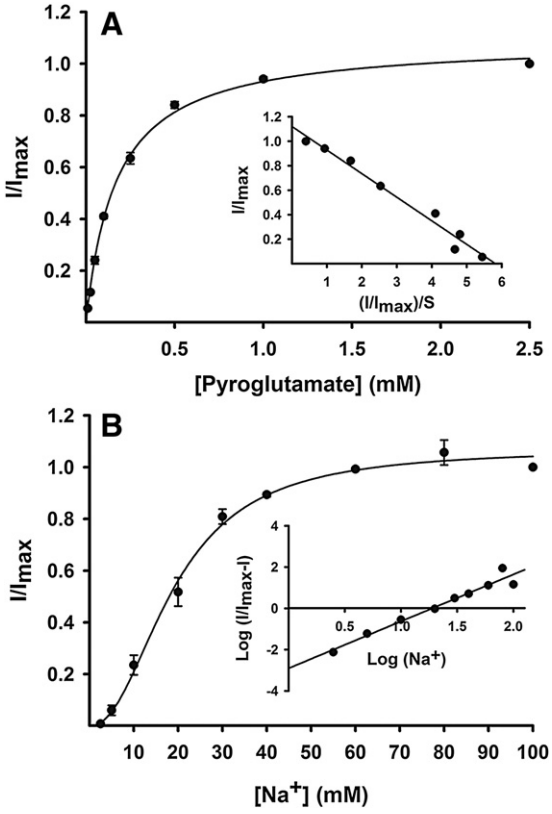


**Fig. 6.** Saturation kinetics (A) and $Na^+$-activation kinetics of SLC5A8-mediated pyroglutamate transport in the *X. laevis* oocyte expression system. (A) Inward currents were monitored in SLC5A8 cRNA-injected oocytes in the presence of increasing concentrations of pyroglutamate in perfusion buffer. The experiment was done in 4 different oocytes. Since the expression levels of SLC5A8 varied to some extent in different oocytes, the data were normalized by taking the maximal current induced by the highest concentration of pyroglutamate (2.5 mM) as 1 in each oocyte and then calculating the currents induced by pyroglutamate at other concentrations as a fraction of this maximal current. Inset: Eadie–Hofstee plot. (B) Pyroglutamate (5 mM)-induced inward currents were monitored in SLC5A8 cRNA-injected oocytes in the presence of increasing concentrations of $Na^+$ (2.5–100 mM). The concentration of $Cl^-$ was maintained at 100 mM by appropriately substituting NaCl with NMDG chloride. The experiment was done with 3 different oocytes, and the currents were normalized as described above to adjust for variations in the expression levels in different oocytes. Inset: Hill plot.

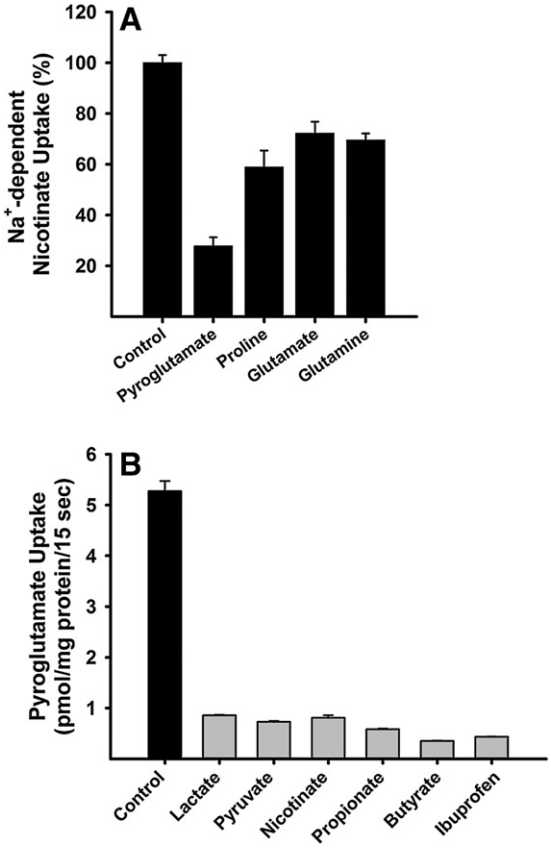


**Fig. 8.** Demonstration of the involvement of SLC5A8 in pyroglutamate uptake in purified rabbit renal brush border membrane vesicles. (A) Uptake [$^{14}$C]nicotinate (25 μM) was measured in renal brush border membrane vesicles in the presence of NaCl or KCl in the uptake medium using a 15 s incubation. Uptake measurements were made in the absence or presence of pyroglutamate, glutamate, glutamine, or proline (5 mM). $Na^+$-dependent uptake, which was calculated by subtracting the uptake in the presence of KCl from the uptake in the presence of NaCl, was taken as a measure of SLC5A8 activity. The transport activity in the absence of inhibitors was taken as 100% (control), and the transport activity in the presence of inhibitors was calculated as percent of this control value. The experiment was repeated with three independent vesicle preparations, and measurements were made in triplicate in each experiment. Data are presented as means ± S. E. (B) Uptake of [$^3$H]-pyroglutamate (1 μM) was measured in renal brush border membrane vesicles with a 15 s incubation in the absence (control) or presence of various substrates or blockers of SLC5A8. When present, the concentration of these compounds was 5 mM. The experiment was repeated with three independent vesicle preparations, and measurements were made in triplicate in each experiment. Data represent means ± S. E.

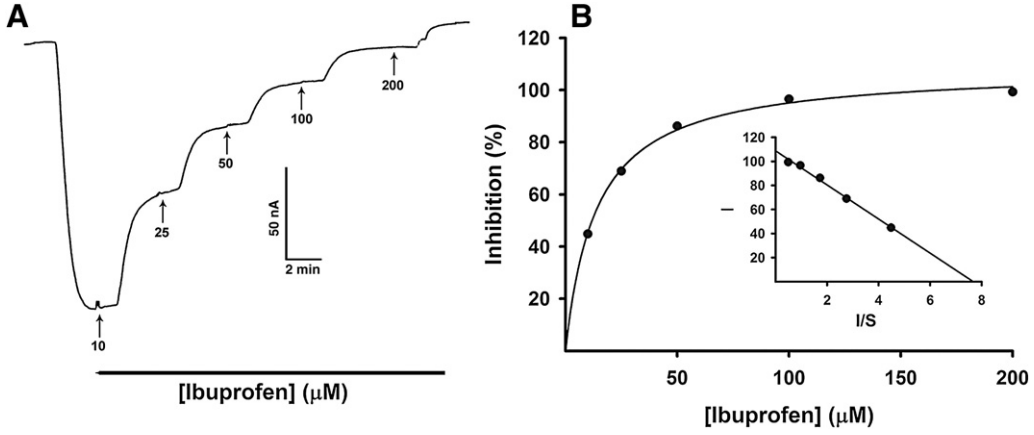


**Fig. 7.** Blockade of SLC5A8-mediated pyroglutamate transport by ibuprofen in *X. laevis* oocytes. (A) Pyroglutamate (1 mM)-induced currents were monitored in SLC5A8 cRNA-injected oocytes in the presence of NaCl and increasing concentrations of ibuprofen. (B) The percent inhibition of pyroglutamate-induced currents by each concentration of ibuprofen was calculated from the above experiment, and the data were used to determine the concentration of ibuprofen needed to cause 50% maximal inhibition by fitting Michaelis–Menten equation to the data. Inset: Eadie–Hofstee plot.

## References


[1] P. Van der Werf, A. Meister, The metabolic formation and utilization of 5-oxo-L-proline (L-pyroglutamate, L-pyrrolidone carboxylate), Adv. Enzymol. Relat. Areas Mol. Biol. 43 (1975) 519–566.

[2] R.F. Doolittle, R.W. Armentrout, Pyrrolidonyl peptidase. An enzyme for selective removal of pyrrolidonecarboxylic acid residues from polypeptides, Biochemistry 7 (1968) 516–521.

[3] R.A. Hawkins, R.L. O'Kane, I.A. Simpson, J.R. Vina, Structure of the blood–brain barrier and its role in the transport of amino acids, J. Nutr. 136 (1 Suppl.) (2006) 218S–226S.

[4] R.A. Hawkins, I.A. Simpson, A. Mokashi, J.R. Vina, Pyroglutamate stimulates $Na^+$-dependent glutamate transport across the blood–brain barrier, FEBS Lett. 580 (2006) 4382–4386.

[5] R.W. Friesen, E.M. Novak, D. Hasman, S.M. Innis, Relationship of dimethylglycine, choline, and betaine with oxoproline in plasma of pregnant women and their newborn infants, J. Nutr. 137 (2007) 2641–2646.

[6] A.A. Jackson, C. Persaud, G. Werkmeister, I.S. McClelland, A. Badaloo, T. Forrester, Comparison of urinary 5-L-oxoprline (L-pyroglutamtate) during normal pregnancy in women in England and Jamaica, Br. J. Nutr. 77 (1997) 183–196.

[7] A. Larsson, M. E. Anderson, Glutathione synthetase deficiency and other disorders of the gamma-glutamyl cycle, in: C. F. Scriver, A. L. Beaudet, W. S. Sly, D. Vallee (Eds.), Metabolic Basis of Inherited Disease, 8th edition, McGrawHill, New York, pp. 2205–2216.

[8] R. Njalsson, S. Norgren, Physiological and pathological aspects of GSH metabolism, Acta Paediatr. 94 (2005) 132–137.

[9] V. Ganapathy, R.A. Roesel, F.H. Leibach, Transport of 5-oxoproline into rabbit renal brush border membrane vesicles, Biochem. Biophys. Res. Commun. 105 (1982) 28–35.

[10] V. Ganapathy, R.A. Roesel, J.C. Howard, F.H. Leibach, Interaction of proline, 5-oxoproline, and pipecolic acid for renal transport in the rabbit, J. Biol. Chem. 258 (1983) 2266–2272.

[11] V. Ganapathy, F.H. Leibach, Electrogenic transport of 5-oxoproline in rabbit renal brush-border membrane vesicles. Effect of intracellular potassium, Biochim. Biophys. Acta 732 (1983) 32–40.

[12] H. Roigaard-Petersen, M.I. Sheikh, Renal transport of amino acids. Demonstration of $Na^+$-independent and $Na^+$-dependent electrogenic uptake of L-proline, hydroxy-L-proline and 5-oxo-L-proline by luminal membrane vesicles, Biochem. J. 220 (1984) 25–33.

[13] E. Gopal, J.J. Fei, M. Sugawara, S. Miyauchi, L. Zhuang, P.M. Martin, S.B. Smith, P.D. Prasad, V. Ganapathy, Expression of slc5a8 in kidney and its role in $Na^+$-coupled transport of lactate, J. Biol. Chem. 279 (2004) 44522–44532.

[14] S. Miyauchi, E. Gopal, Y.J. Fei, V. Ganapathy, Functional identification of SLC5A8, a tumor suppressor downregulated in colon cancer, as a $Na^+$-coupled transporter for short-chain fatty acids, J. Biol. Chem. 279 (2004) 13293–13296.

[15] M.J. Coady, M.H. Chang, F.M. Charron, C. Plata, B. Wallendorff, J.F. Sah, S.D. Markowitz, M.F. Romero, J.Y. Lapointe, The human tumour suppressor gene SLC5A8 expresses a $Na^+$-monocarboxylate cotransporter, J. Physiol. 557 (2004) 719–731.

[16] V. Paroder, S.R. Spencer, M. Paroder, D. Arango, S. Schwartz Jr., J.M. Mariadason, L. H. Augenlicht, S. Eskandari, N. Carrasco, $Na^+$/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: molecular characterization of SMCT, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 7270–7275.

[17] . P.M. Martin, E. Gopal, S. Ananth, L. Zhuang, S. Itagaki, B.M. Prasad, S.B. Smith, P. D. Prasad, V. Ganapathy, Identity of SMCT1 (SLC5A8) as a neuron-specific $Na^+$-coupled transporter for active uptake of L-lactate and ketone bodies in the brain, J. Neurochem. 98 (2006) 279–288.

[18] E. Gopal, Y.J. Fei, S. Miyauchi, L. Zhuang, P.D. Prasad, V. Ganapathy, Sodium-coupled and electrogenic transport of B-complex vitamin nicotinic acid by slc5a8, a member of the $Na^+$/glucose cotransporter gene family, Biochem. J. 388 (2005) 309–316.

[19] S. Itagaki, E. Gopal, L. Zhuang, Y.J. Fei, S. Miyauchi, P.D. Prasad, V. Ganapathy, Interaction of ibuprofen and other structurally related NSAIDs with the sodium-coupled monocarboxylate transporter SMCT1 (SLC5A8), Pharm. Res. 23 (2006) 1209–1216.

[20] E. Gopal, S. Miyauchi, P.M. Martin, S. Ananth, P. Roon, S.B. Smith, V. Ganapathy, Transport of nicotinate and structurally related compounds by human SMCT1 (SLC5A8) and its relevance to drug transport in the mammalian intestinal tract, Pharm. Res. 24 (2007) 575–584.

[21] E. Gopal, N.S. Umapathy, P.M. Martin, J.P. Gnanaprakasam, H. Becker, C.A. Wagner, V. Ganapathy, P.D. Prasad, Cloning and functional characterization of human SMCT2 (SLC5A12) and expression pattern of the transporter in kidney, Biochim. Biophys. Acta 1768 (2007) 2690–2697.

[22] M. Thangaraju, S. Ananth, P.M. Martin, P. Roon, S.B. Smith, E. Sterneck, P.D. Prasad, V. Ganapathy, c/ebpδ null mouse as a model for the double-knockout of slc5a8 and slc5a12 in kidney, J. Biol. Chem. 281 (2006) 26769–26773.

[23] H. Frank, N. Groger, M. Diener, C. Becker, T. Braun, T. Boettger, Lactaturia and loss of sodium-dependent lactate uptake in the colon of SLC5A8-deficient mice, J. Biol. Chem. 283 (2008) 24729–24737.

[24] M. Barac-Nieto, H. Murer, R. Kinne, Lactate-sodium cotransport in rat renal brush border membranes, Am. J. Physiol. 239 (1980) F496–F506.

[25] K.E. Jorgensen, M.I. Sheikh, Renal transport of monocarboxylic acids. Heterogeneity of lactate-transport systems along the proximal tubule, Biochem. J. 223 (1984) 803–807.

[26] B. Barbarat, R.A. Podevin, Stoichiometry of the renal sodium-L-lactate cotransporter, J. Biol. Chem. 263 (1988) 12190–12193.

[27] R. Mengual, M.H. Schlageter, P. Sudaka, Kinetic asymmetry of renal $Na^+$-L-lactate cotransport. Characteristic parameters and evidence for a ping pong mechanism of the trans-stimulating exchange by pyruvate, J. Biol. Chem. 265 (1990) 292–299.

[28] S.E. Guggino, P.S. Aronson, Paradoxical effects of pyrazinoate and nicotinate on urate transport in dog renal microvillus membranes, J. Clin. Invest. 76 (1985) 543–547.